Sodium Dependent Vitamin C Transporters in the Sheep Corpus Luteum: Sequence Analysis

Ryan P. Ceddia, Macdonald P. Wick, and Joseph S. Ottobre

Department of Animal Sciences
The Ohio State University

Abstract

Vitamin C is a multifunctional antioxidant that is sequestered within the corpus luteum (CL). The concentrations of vitamin C found in the functional CL are 50-100 fold greater than concentrations in systemic plasma. Vitamin C may play a protective antioxidant role in the CL and has been shown to prevent apoptosis. The loss of vitamin C from the CL has been associated with luteolysis. Two sodium dependent vitamin C transporters (SVCT1 and SVCT2) have been described in various species, and the primary sequences of these proteins are known (e.g., human, pig, guinea pig, rats, mice). The primary sequences for SVCT1 and SVCT2 have not yet been reported in the sheep. Vitamin C transporters are highly conserved, and it is likely that the sheep CL expresses similar transport proteins. Since these transport proteins would represent critical elements in the regulation of vitamin C concentrations in the CL, the objective of the current work was to determine the primary protein sequences for SVCT1 and SVCT2 in the sheep. CL were surgically collected from regularly cycling sheep on day 3 of the estrous cycle. Luteal tissue was immediately snap frozen in liquid nitrogen and stored at -80° C. RNA was extracted from CL and transcribed into cDNA using MMLV reverse transcriptase. PCR was then run using primers that were designed from known SVCT sequences. PCR-amplified cDNA fragments of the predicted lengths were resolved by agarose electrophoresis, excised, and cloned into plasmids. Plasmids were transformed into *Escherichia coli* for amplification prior to DNA sequence analyses. We have sequenced a 296 base pair portion of the message for ovine SVCT1 and an 1860 base pair portion of the message for ovine SVCT2. This encodes for 98 amino acids for oSVCT1 and 618 amino acids plus the stop codon for oSVCT2. The length of the oSVCT1 amino acid sequence corresponds to 14% of the presumptive sequence based upon that of the human. We have found the sheep message to have high homology with that of the human (93%), the pig (92%), the rat (90%), and mouse (90%). The protein sequence was also found to have

high homology with that of the human (100%), the pig (98%), the rat (100%), and mouse (98%). The length of the oSVCT2 amino acid sequence corresponds to 95% of the presumptive sequence based upon that of the human. We have found the sheep message to have high homology with that of the human (90%), the pig (93%), the rat (87%), and the mouse (87%). The protein sequence was also found to have high homology with that of the human (89%), the pig (88%), the rat (86%), and mouse (86%). These data are important in furthering studies of the regulation of SVCT proteins and vitamin C concentrations in the CL of the sheep.

Introduction

The functional corpus luteum (CL) is an ovarian structure that produces progesterone and is important for the maintenance of pregnancy in the sheep. The CL, along with several other tissues such as the adrenal glands, brain, and pituitary, is known to have high concentrations of L-ascorbic acid, vitamin C (Szent-Györgyi, 1928; Glick and Biskind, 1935a; Glick and Biskind, 1935b; Biskind and Glick, 1936; Levine and Morita, 1985; Petroff et al., 1997). The concentration of vitamin C in the CL is the highest in the body, with the exception of the adrenal glands and pituitary, and is hormonally regulated (Paeschke, 1970 [cited in Pepperell et al., 2003]; Levine and Morita, 1985; Musicki et al., 1996; Petroff et al., 1997). The vitamin C content in the CL is at its maximum when the CL is fully mature, remains high during pregnancy, and decreases as the CL regresses (Biskind and Glick, 1936; Hoch-Ligeti and Bourne, 1948; Petroff et al., 1997).

In the absence of pregnancy in the sheep, luteal regression begins on day fourteen after estrus and continues through days sixteen and seventeen (Rothchild, 1981; Hoyer, 1998). Uterine prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) is the luteolytic signal in ruminants and other mammals (Rothchild, 1981; Horton and Poyser, 1976; Behrman et al., 1993; Niswender and Nett, 1994; Niswender et

al., 2000). $PGF_{2\alpha}$ can shorten the estrous cycle or end pregnancy by inducing luteolysis (i.e., regression of the CL), thus ending the supply of progesterone. The decrease in progesterone precedes structural luteolysis and is followed by the loss of luteal weight around twenty-four hours after exposure to $PGF_{2\alpha}$ (Christenson et al., 1995; Hoyer, 1998). In a young CL, in the case of sheep prior to days three through five of the estrous cycle, $PGF_{2\alpha}$ has a transient effect (Rothchild, 1981; Pope and Cárdenas, 2004). There have been several proposed mechanisms to explain the luteolytic effect of $PGF_{2\alpha}$: a rapid decrease in luteal blood flow, a reduced number of luteinizing hormone receptors, an uncoupling of luteinizing hormone receptors from adenylate cyclase, the activation of protein kinase C, an influx of calcium, generation of oxidative radicals, and a cytotoxic effect (Horton and Poyser, 1976; Behrman et al., 1993; Niswender and Nett, 1994; Tilly, 1996; Niswender et al., 2000). These events have been shown to occur in response to $PGF_{2\alpha}$, but it is important to consider their sequence and to determine which events are consequences of others.

In the CL, oxidative radicals cause lipid peroxidation, decreased membrane fluidity, calcium influx, desensitization of the luteinizing hormone-receptor complexes, loss of steroidogenesis, inhibition of RNA synthesis, and RNA and genomic degradation; all of these events are consistent with apoptosis, programmed cell death, thus suggesting that oxidative radicals may be fundamental in the initiation of luteolysis and therefore inhibition of progesterone synthesis (Sawada and Carlson, 1985; Behrman et al., 1989; Sawada and Carlson, 1989; Margolin et al., 1990; Riley and Behrman, 1991; Sawada and Carlson, 1991; Aten et al., 1992; Behrman et al., 1993; Carlson et al., 1993; Musicki et al., 1994; Sawada and Carlson, 1994; McConkey and Orrenius, 1996; Tilly, 1996; Kato et al., 1997; Dharmarajan et al., 1999; Niswender et al., 2000; Tanaka et al., 2000; Pepperell et al., 2003). There are several

mechanisms by which cells protect themselves from oxidative radicals, including antioxidant vitamins, such as vitamin C (Biskind and Glick, 1936; Aten et al., 1992, Behrman et al., 1993; Kato et al., 1997; Tanaka et al., 2000). Vitamin C, in the forms of L-ascorbic acid and semidehydroascorbic acid, the free radical form, reduce oxidative radicals by donating one electron and in the process are converted to semidehydroascorbic acid or dehydroascorbic acid, respectively (Szent-Györgyi, 1928; Sawyer et al., 1982; Winkler et al., 1994; Levine and Morita, 1985; Bendich et al., 1986; Niki, 1991; Rose and Bode, 1993). Vitamin C is especially important biologically, because one molecule can deactivate more than 2.5 superoxide ions and its one-electron reduction potential is lower than many other antioxidants (Sawyer et al., 1982; Rose and Bode, 1993). Both vitamin C and vitamin E have been shown to suppress apoptosis (Vierk et al., 1988; Tilly and Tilly, 1995; McConkey and Orrenius, 1996; Dharmarajan et al., 1999). Vitamin E, which vitamin C can reduce, thereby restoring its antioxidant properties, is the only lipid soluble antioxidant. This feature makes Vitamin E especially important in the reduction of oxidative radicals in hydrophobic areas (Golumbic and Mattill, 1941; Chen et al.,, 1980; Packer et al., 1979; Bendich et al., 1986; Niki, 1987; Niki, 1991; Winkler et al., 1994). Besides generating oxidative radicals, $PGF_{2\alpha}$ stimulates vitamin C depletion. This is due to stimulation of cellular secretion and by inhibition of vitamin C uptake (Stansfield and Flint, 1967; Fomichev, 1971 [cited in Pepperell 2003]; Sato et al., 1974; Levine and Morita, 1985; Aten et al., 1992; Musicki et al., 1996; Petroff et al., 1998; Tsai and Wiltbank, 1998). In pigs, treatment of CL with $PGF_{2\alpha}$ increased the concentrations of vitamin C in the veins draining the ovary within fifteen minutes (Petroff et al., 1998). More than fifty-percent of the vitamin C depletion occurs in the first 2.5 minutes with the depletion remaining for up to two hours (Musicki et al., 1996). This suggests that vitamin C efflux is one of the earliest events in luteolysis, as an increase in

apoptosis does not occur until progesterone concentrations decrease (Juengel et al., 1993). CL that are exposed to $PGF_{2\alpha}$ prior to day 3 of the ovine estrous cycle remain functional and regain vitamin C, whereas CL that are exposed to $PGF_{2\alpha}$ on day 10 do not regain the vitamin C that is lost and ultimately regress (Gaddis and Ottobre, unpublished data). In the CL, concentrations of vitamin C increase as the CL ages; vitamin C concentrations rise from around 440 μg/g in the day 3 CL to around 670 μg/g in the day 10 CL (Gaddis and Ottobre, unpublished data).

Vitamin C is transported into many tissues, including the CL, via an energy- and sodium-dependent process carried out by two sodium-dependent vitamin C transporters: SVCT1 and SVCT2 (Tsukaguchi et al., 1999). Portions of SVCT1 have been sequenced in the human, pig, rat, mouse, and guinea pig, while portions of SVCT2 have been sequenced in the dog and rabbit, in addition to the species mentioned for SVCT1. Northern-Blot analysis shows that ovarian tissue has large amounts of SVCT1 and SVCT2 mRNA, thus suggesting that these transport proteins are important in maintaining the large amounts of vitamin C found in the CL (Wang et al., 1999). SVCT2 is widely distributed with a 7.5 kb transcript detected in most tissues, except lung and skeletal muscle (Rajan et al., 1999; Tsukaguchi et al., 1999; Wang et al., 2000; Clark et al., 2002). SVCT1 is highly expressed in kidney, liver, small intestine, colon, prostate, and ovary with approximately 2.4 – 3 kb transcript; this corresponds with tissues that have large quantities of Vitamin C (Tsukaguchi et al., 1999; Wang et al., 1999, Wang et al., 2000). SVCT1, the primary role of which appears to be in the absorption of dietary Vitamin C, has a higher V_{max} than SVCT2, resulting in a higher influx of vitamin C (Tsukaguchi et al., 1999; Liang et al., 2001; Takanaga et al., 2004). We suspect that the level of expression of the SVCT1 and SVCT2 genes in the CL may change as concentrations of Vitamin C change during luteal development and

regression. We also suspect that treatment with $PGF_{2\alpha}$ may result in divergent effects on the expression of SVCT1 and SVCT2 that depend on the age of the CL at the time of treatment.

As mentioned above, the primary sequences for SVCT1 and SVCT2 have not yet been reported in the sheep. Vitamin C transporters are highly conserved, and it is likely that the sheep CL expresses similar transport proteins. Since these transport proteins would represent critical elements in the regulation of vitamin C concentrations in the CL, the objective of the current work was to determine portions of the nucleotide sequences for SVCT1 and SVCT2 in the sheep.

Materials & Methods

Experimental model and collection of previously obtained CL:

Commercial ewes (n = 22) were used in this study. The onset of estrus (day 0) was determined by twice daily observation in the presence of a vasectomized ram. Surgical procedures were performed on day 3 (early luteal phase) or day 10 (mid luteal phase). Intact ewes received intramuscular injections of 25mg of $PGF_{2\alpha}$ (Lutalyse; Pharmacia & Upjohn, Kalamazoo, MI) or vehicle on day 3, a time when CL are insensitive to the luteolytic effects of $PGF_{2\alpha}$ or day 10, a time when CL are sensitive to the luteolytic effects of $PGF_{2\alpha}$. Ewes were randomly assigned to one of four groups: early luteal phase control (n = 5), early luteal phase $PGF_{2\alpha}$-treated (n = 7), mid luteal phase control (n = 5), and mid luteal phase $PGF_{2\alpha}$-treated (n = 5). On day 3 or 10 of the estrous cycle, depending on the experimental group, ewes were transported to the surgical facility (Figure 1). Prior to the first surgery, one jugular vein was catheterized using a 14 g x 5.5” i.v. catheter (Abboth Ireland, Republic of Ireland). Ewes were sedated using intravenous Pentothal (Abbott Laboratories, North Chicago, IL) in order to intubate them using an Aire-Cuf veterinary endotracheal tube (i.d. 10 mm, o.d. 14.3 mm; Bivona Inc., Gary, IN). A surgical plane of anesthesia was maintained throughout the experiment using

halothane gas. A midventral laparotomy was performed to expose the reproductive tract. Each ewe received an intramuscular injection of either 25mg of $PGF_{2\alpha}$ or saline (time 0). One CL was removed at 2 hours post-treatment. At twenty-four hours post-treatment, a surgical plane of anesthesia was established and the remaining CL were removed.

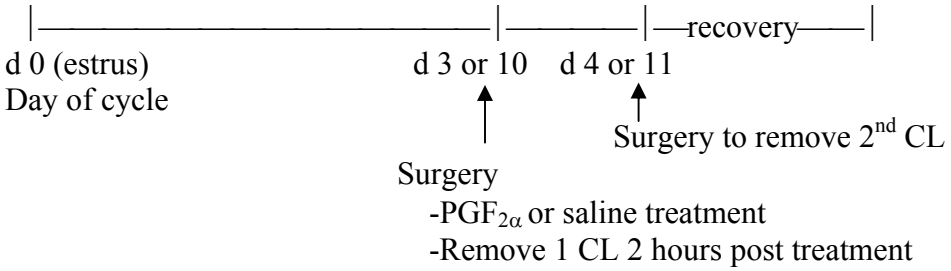


Figure 1. Experimental Protocol for Control and $PGF_{2\alpha}$ Treated Ewes

Luteal tissues were immediately dissected into three portions and snap frozen in liquid nitrogen. The samples were stored at -80°C. One portion was used to measure progesterone concentrations, and another to measure vitamin C concentrations which was used for the Gaddis and Ottobre (unpublished data). The third portion of the CL was used for RNA isolation and gene sequencing. (This portion of materials and methods was adapted from Gaddis and Ottobre unpublished data).

RNA isolation and gene sequencing:

The RNA was isolated from a day 3 control CL using RNAqueous®-4PCR Kit (Ambion Inc., Austin, TX). Total RNA was then reverse transcribed with RETROscript® Kit (Ambion Inc., Austin, TX), which utilizes the MMLV reverse transcriptase to create the cDNA. The cDNA generated was then run in a series of polymerase chain reactions (PCR). A variety of primers and conditions were used in order to amplify PCR targets. Modifications for PCR included varying thermal cycling conditions, concentration of Taq DNA polymerase, and running PCR on PCR

product cDNA excised from agarose gel. Herculase® Enhanced DNA polymerase (Stratagene, La Jolla, CA) was utilized in all PCR reactions.

PCR primer design:

Two sets of overlapping primers were used to isolate SVCT2 fragments: 5' CTTTACTCTTCCGGTGGTG 3' (forward) & 5' ATGCCATCGAGAACACAGG 3' (reverse), and 5' GTCTATCGGGGACTACTACGCC 3' (forward) & 5' GGCTATACTGTGGCCTGGG 3' (reverse). These sets of primers were anticipated to yield 1196 base pair and 776 base pair fragments respectively. The primers were similar to those used by Clark et al. (2002), in the guinea pig, and Gispert et al. (2000), in the mouse. The primers 5' GTCTATCGGGGACTACTACGCC 3' (forward) & 5' ATGCCATCGAGAACACAGG 3' (reverse) also were used to amplified the 114 base pair overlap.

Sodium Dependent Vitamin C Transporter Sequences							
	Homo sapiens	*Sus scrofa*	*Rattus norvegicus*	*Mus musculus*	*Cavia porcellus*	*Canis familiaris*	*Oryctolagus cuniculus*
SVCT1	AF170911	AY353718	AF080452	BC013528	AF410935		
SVCT2	AJ269478	AF058320	AF080453	AY004874	AF411585	AY264779	AF118561

Table 1. GenBank reference numbers for sequences of SVCT1 and SVCT2 used to design PCR primers for the respective sequences in the sheep.

The SVCT1 primers, 5' CACTGGAGAGATGGAAATGCC 3' (forward) & 5' GATCAGGAGAATGGAGCAAGC 3' (reverse) were designed to be one-hundred percent homologous to the pig sequence AY353718 and were expected to yield a 296 base pair fragment. This was because the SVCT2 sequence was most similar to the pig. Primers were manufactured by Invitrogen (Carlsbad, CA).

Polymerase Chain Reaction:

All SVCT2 primer combinations that were reported to work required PCR conditions of 1 minute at 94º C, 2 minutes at 55º C, and 3 minutes at 68º C. SVCT1 required PCR conditions of 1 minute at 94º C, 2 minutes at 60º C, and 3 minutes at 72º C with a reduced amount of *Taq* DNA polymerase.

Cloning and Sequencing:

Following PCR, the product was run on an agarose gel and bands were visualized using UV light following staining with ethidium bromide. cDNA from bands corresponding to the expected sizes was then extracted using QIAquick Gel Extraction® (Qiagen, Valencia, CA). For SVCT2 the gel extract was used for cloning, but for SVCT1, the sample was re-run under identical PCR conditions and the PCR product was cloned. The product was then cloned into *Escherichia coli* using TOPO TA Cloning® Kit For Sequencing (Invitrogen, Carlsbad, CA). The plasmids were then purified using Qiagen Midi Plasmid Prep Kit (Qiagen, Valencia, CA). The plasmids were then sent to Genotyping Sequencing Unit in the Comprehensive Cancer Center at The Ohio State University for sequence analysis.

Sequencing and data analysis:

Plasmid samples were sequenced using the M13 forward and reverse primers. BigDye sequencing chemistry was used on an ABI3700 and performed by the Genotyping Sequencing Shared Resource at The Ohio State University. Sequence identity was confirmed by Basic Local Alignment Search Tool (BLAST) utilizing programs provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). DNA sequence was translated into protein sequence using the Translate tool provided by Expert

Protein Analysis System (ExPASy) Proteomics Server (http://us.expasy.org/). Hydropathy analysis was performed using the WinPep 3.01 program.

Results

We have sequenced a 296 base pair portion of the message for ovine SVCT1 and an 1860 base pair portion of the message for ovine SVCT2. The first sequence encodes for 98 amino acids for oSVCT1 acids and the second encodes for 618 amino acids plus the stop codon for oSVCT2 (Figure 2). The length of the oSVCT1 sequence corresponds to 14% of the presumptive sequence and the oSVCT2 sequence corresponds to 95% of the presumptive sequence based upon that of the human. When compared to the amino acid sequence for SVCT2 in Tsukaguchi et al. (1999), the oSVCT2 sequence contains the first methionine as well as additional five-prime sequence. The oSVCT2 sequence contains all twelve putative membrane spanning regions.

Ovine Sodium Dependent Vitamin C Transporter 1

DNA Sequence

GATCAGGAGAATGGAGCAAGCTGAGATACCCCAGTGGGAGCCCGCTCGATCGCCAGCAGCTTGG
AAGACAGAGAGACCAATGAGGGAGACAGTGGGGGTGACTGTAAGAGGCCCAATGTAACTGAGCA
GGGCCCCAGGCAGCCCCATCAGCCCAATCACCACCTCCACCATACTGGACACCATGATTGCACC
CTGGACCTCTCGTATCCGTGGGTGCCAAATATGTGAGGTATTCAGGGGCAGACTCCAGTTACCG
TAGATCTCTTCTTCTGGAGGGCATTTCCATCTCTCCAGTG

296 Base Pairs

Protein Sequence

LERWKCPPEEEIYGNWSLPLNTSHIWHPRIREVQGAIMVSSMVEVVIGLMGLPGALLSYIGPLT
VTPTVSLIGLSVFQAAGDRAGSHWGISACSILLI

98 Amino Acids

Ovine Sodium Dependent Vitamin C Transporter 2

DNA Sequence

CTTTACTCTTCCGGTGGTGATCAATGGAGGGGCCACGTCCAGTGGTGAACAGGACAATGAAGAT
ACTGAGCTCATGGCAATATATACAACAGAAAACGGCATCGCAGAAAAGAGCTCCCTTGCTGAGA
CCTTGGATAGCACTGGCAGTCTAGACCCCCAGAGATCAGATATGATTTACACCATAGAAGATGT
TCCACCCTGGTACCTGTGCATATTTCTGGGGTTGCAGCATTATCTGACATGTTTTAGCGGCACC

ATTGCAGTGCCCTTTCTGCTGGCCGACGCCATGTGTGTGGGATATGACCAGTGGGCCACCAGCC
AGCTCATTGGGACCATTTTCTTCTGCGTGGGAATCACGACTTTGCTGCAGACTACTTTTGGATG
CAGGTTACCCCTGTTTCAGGCCAGTGCTTTTGCATTTCTGGCCCCTGCTCGAGCCATCCTGTCT
TTAGATAAATGGAAATGTAACACCACAGATGTTTCGATTGCCAATGGAACAACAGAGCTGTTAC
ACACAGAACACATCTGGTATCCCCGAATACGCGAGATCCAGGGAGCCATCATCATGTCCTCATT
GATAGAAGTGGTCATCGGCCTCCTAGGCCTGCCTGGGGCTCTGCTGAAATACATCGGGCCCCTG
ACCATCACACCCACGGTAGCCCTCATTGGCCTCTCTGGTTTCCAGGCAGCAGGAGAGAGAGCAG
GGAAGCACTGGGGCATCGCCATGCTGACTATTTTCCTAGTATTACTGTTTTCTCAATATGCCAG
AAATGTTAAATTTCCTCTCCCAATTTACAAATCCAAGAAAGGATGGACTGCATATAAGTTACAG
CTTTTCAAAATGTTCCCTATCATCCTGGCCATCCTTGTGTCCTGGCTACTCTGCTTCATCTTCA
CAGTGACAGATGTCTTCCCTCCTGACAGCACGAAGTATGGCTTCTATGCTCGAACTGATGCCAG
GCAGGGTGTGCTGCTGGTAGCCCCGTGGTTTAAGGTCCCATACCCATTTCAGTGGGGACTGCCC
ACCGTCTCTGCAGCTGGTGTCATCGGCATGCTCAGTGCGGTTGTTGCCAGTATTATCGAGTCTA
TTGGGGACTACTATGCCTGTGCAAGGTTGTCTTGTGCCCCGTCACCTCCCATCCATGCAATAAA
CAGGGGGATTTTCGTGGAGGGTCTCTCCTGTGTTCTCGATGGCATTTTTGGTACTGGGAATGGC
TCTACTTCATCCAGTCCCAACATTGGAGTTTTGGGAATTACTAAGGTTGGCAGTCGTCGGGTGA
TACAGTACGGCGCAGCCCTCATGCTCGCATTGGGCATGATCGGAAAGTTCAGTGCCCTCTTCGC
CTCCCTTCCAGATCCTGTGCTTGGTGCCCTCTTCTGTACTCTCTTTGGAATGATCACAGCTGTT
GGACTCTCTAACTTGCAGTTCATTGATTTAAATTCTTCCCGGAACCTCTTTGTGCTTGGATTTT
CAATCTTCTTTGGGCTCGTCCTTCCAAGTTACCTCAGACAGAACCCTCTTGTCACAGGGATAAC
AGGAATCGATCAAGTGTTGAATGTTCTTCTCACAACTGCTATGTTTGTAGGAGCCTGTGTGGCT
TTTATTTTGGATAACACCATCCCAGGTACTCCAGAGGAAAGAGGAATCAGAAAATGGAAGAAGG
GCGTGGGCAAAGGGAGCAAGTCTCTCGATGGCATGGAATCCTATGATTTACCATTTGGCATGAA
CATTATTAAAAAATACAGATGTTTCAGCTACTTACCCATCAGCCCAACCTTTGCAGGCTACACA
TGGAAAGGCCTCGGGAAGAGCGCTAGTAGCCGGAGTTCAGATGAAGACTCACAGGCCACAGTAT
AGCC

1860 base pairs

Protein Sequence

FTLPVVINGGATSSGEQDNEDTELMAIYTTENGIAEKSSLAETLDSTGSLDPQRSDMIYTIEDV
PPWYLCIFLGLQHYLTCFSGTIAVPFLLADAMCVGYDQWATSQLIGTIFFCVGITTLLQTTFGC
RLPLFQASAFAFLAPARAILSLDKWKCNTTDVSIANGTTELLHTEHIWYPRIREIQGAIIMSSL
IEVVIGLLGLPGALLKYIGPLTITPTVALIGLSGFQAAGERAGKHWGIAMLTIFLVLLFSQYAR
NVKFPLPIYKSKKGWTAYKLQLFKMFPIILAILVSWLLCFIFTVTDVFPPDSTKYGFYARTDAR
QGVLLVAPWFKVPYPFQWGLPTVSAAGVIGMLSAVVASIIESIGDYYACARLSCAPSPPIHAIN
RGIFVEGLSCVLDGIFGTGNGSTSSSPNIGVLGITKVGSRRVIQYGAALMLALGMIGKFSALFA
SLPDPVLGALFCTLFGMITAVGLSNLQFIDLNSSRNLFVLGFSIFFGLVLPSYLRQNPLVTGIT
GIDQVLNVLLTTAMFVGACVAFILDNTIPGTPEERGIRKWKKGVGKGSKSLDGMESYDLPFGMN
IIKKYRCFSYLPISPTFAGYTWKGLGKSASSRSSDEDSQATV**stop**

618 Amino Acids

Figure 2. DNA and Protein sequence of SVCT1 and SVCT2 from the sheep. Putative membrane spanning regions, based upon those found in Tsukaguchi et al. (1999), are underlined in the amino acid sequence. The oSVCT1 sequence spans from the last amino acid in putative membrane spanning region 3 and continues to putative membrane spanning region 5. The oSVCT2 sequence contains all twelve putative membrane spanning regions.

The SVCT sequences appear to be highly conserved and have high homology when compared to all currently known sequences (Tables 2 and 3). The SVCT2 homology comparisons are more accurate than the SVCT1 due to the larger number of nucleotides/amino acids that have been sequenced. The sequences are also highly conserved when compared to each other. The high level of homology of oSVCT1 with other species is related to the fact that primers were designed from regions of high homology, and the length of the sequence that we determined is limited. The SVCT2 sequence from the guinea pig is also limited, which may partially account for the high homology with the sheep sequence.

Table 2:

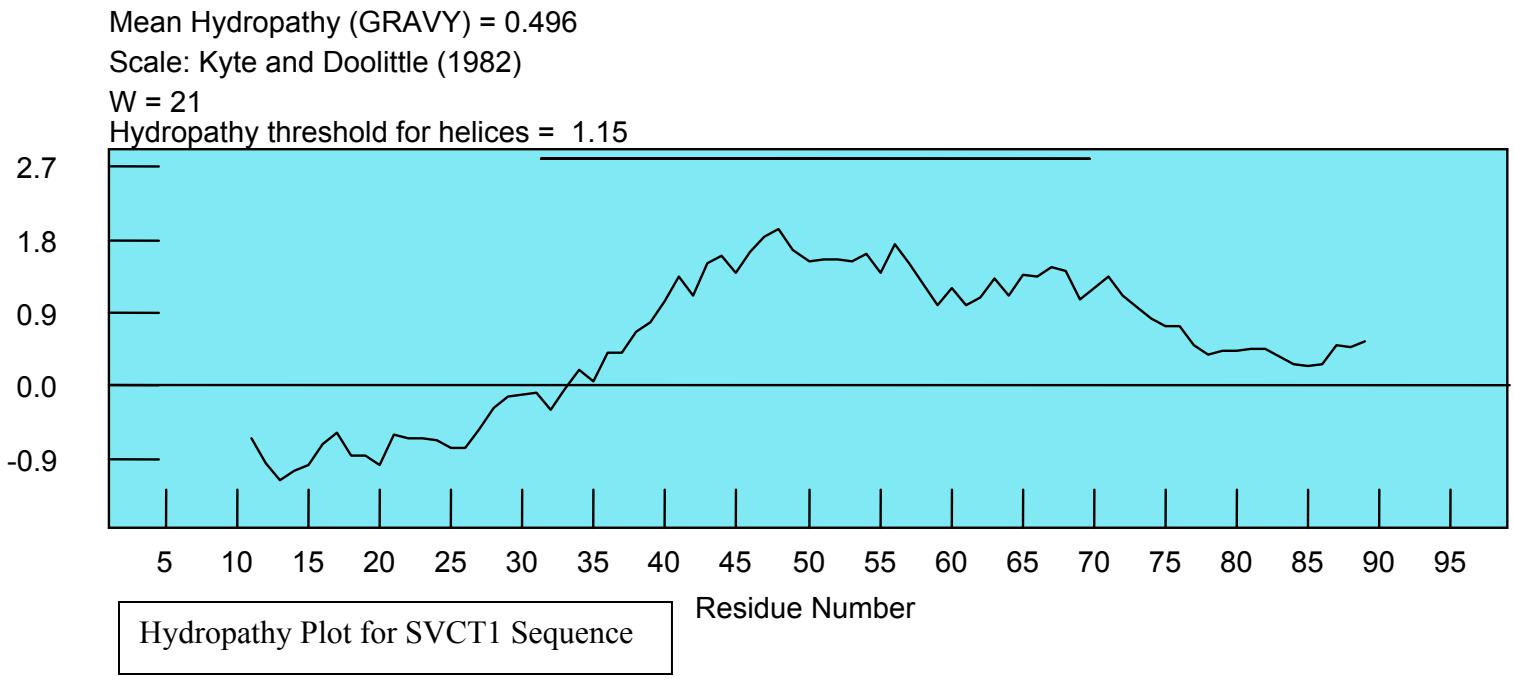
Ovine Sodium Dependent Vitamin C Transporter Comparisons								Nucleotide
	Human	Pig	Rat	Mouse	Guinea Pig	Dog	Rabbit	Sheep - opposite SVCT sequence
SVCT1	93%	92%	90%	90%	91%			73%
SVCT2	90%	93%	87%	87%	92%	93%	89%	73%

Table 3:

Ovine Sodium Dependent Vitamin C Transporter Comparisons								Amino Acid
	Human	Pig	Rat	Mouse	Guinea Pig	Dog	Rabbit	Sheep - opposite SVCT sequence
SVCT1	100%	98%	100%	98%	100%			63%
SVCT2	89%	88%	86%	86%	97%	87%	89%	63%

Tables 2 & 3. Comparisons of the ovine SVCT1 and SVCT2 nucleotide and amino acid sequences across species.

The hydropathy analysis confirms the presence of twelve putative membrane domains (Figure 3). This provides further evidence that the sequences we have cloned are bona fide SVCT1 and SVCT2 sequences. The hydropathy analysis confirms that the proteins encoded by these genes are transmembrane proteins. Also, relatively few proteins have twelve transmembrane domains.



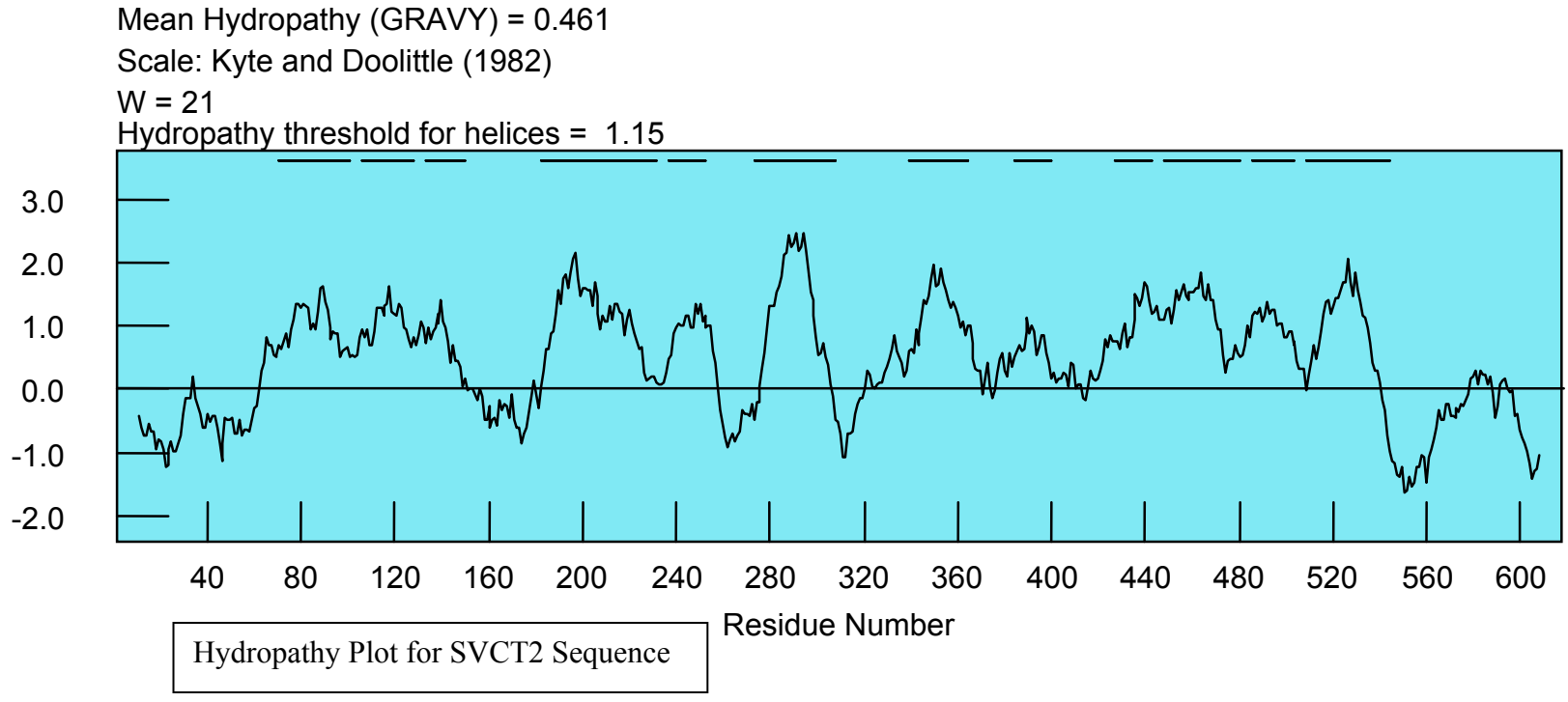


Figure 3. Kyte-Doolittle hydrophobicity plots of oSVCT1 (top) and oSVCT2 (bottom) amino acid sequences. These plots were generated using a 21-amino-acid window. The hydrophobicity threshold for transmembrane helices was set at 1.15. Bars above the hydrophobicity peaks represent the putative transmembrane regions. Note the presence of twelve putative membrane domains in SVCT2. Hydrophobicity analysis was performed using the WinPep 3.01 program.

Other sequences obtained:

The primers that were designed to amplify SVCT sequences were not specific for the target sequence. As a result, other sequences were amplified during PCR. Several of these sequences were cloned and sequenced (Figure 4).

A.

CGGCTGCCCACCTTGGGAAAAAAAGAGTTTTTTCTCTTCCACCAAGAAAAGACAAGTTCAGAGC
CTGATTGTCTTTGGCCTGAATTGAGTCACATGATGACTCAGGAAGAAAGCCTGTGTTCACTGAG
TGCGACTTCCTGAAGCTGGAGGTGGGATCAGTCCTGCGTGAGCCGGGATGGGGGAAGGGTGATG
GTGCCCAAGACCCGCGATTAGGAGTGGGTGGCCCAAGGAGCCATCCCTGCCTGTCAATTGGGAC
CGGGTTAACCCGCGTGGGAGAGCGCGGGTGGTTCTTGGACAGAGGTATGTCTGGACGCCTGTGC
CCCCCGGGGCCGAGGGAGGGAGGGGAATACACAGGTGTGGACAGGCAGACGCTGGAGGATTTGT
TCAGAGAGAGCACAGTGGTGCCACCTCGGGGGGGCATCATATGGGAGGCAGTGGATGTGGGGGA
ACGGGGTCCCCTTGTTACCTGGGAACAGCTAACCTGCAGAGCCTAACAAGGCCTGCCCAGCAGT
ACCGGATTTCCCCAGTGCTTTGACACCACAAGGCTTTCCATGCCTCGTGTCCCTGGATCTTCCC
CATCTTCCTGCGCAGCAGGTCGGGTTGTTACCCTGAGGATCTCCTTTCAACAGGTGAGGTACCA
CAGAGCAGGCACCCTGGCCTCCTCTGGGGCCAAGCTGTGTGGAATGGAAGCTGGGCTCTGCTGT
GGGACTTCAGNCCAGACGCTCAGCCTCTCTGTGCCTTGGCCTCTGTGCTTGTAAGAGGAGCTGA
TGGCAGTGCCCAAAGCACAGGGTGTGGCGAGGCTGAGACATGTTGGTGACATGAGGTCGCCACT
TGGCGTGCTGCAGGGAGATCCGGGGTTGGCCGGCTGGATTCAATCCAGCAGCCGGCCCAGTGGT
TTCCAGACCCCACGGCTGTGTGCCGGGAAACTTTGGATAAACCTTTCCATGGAAGTCTCTAGTG
CTCTCTTGTGAACACTCCTTCTCGCAGCCAGGACTGCAAGCAAGACAACGACCCTGCGGGACTG
CCTGGCTGACGCTGAGCTGGTGAGGCCGCCCGGCACTCTTATTAAACAGACTGGCTGATGCTGC
CAATCTGAGCCGAGCCAGGGATGCATGACCTGGAGGTCTTTCTCCGGCTGCTTCCGCCTGGGTC
AAGGCCTCCAGGGGTGGAGTGCTGAGGGTTTGGGCTTCTCTTCTTTGAGGGGAAACATGGGGAA
TGGCAGAAGGAGGTGGTTTGCGCAGTGGACTGATTGAAGCGGGTTGTTTGCCTGCCGTAAAGGG
GGAGACCTTCGAGCTTGGCTATCCAAGGTGGGCAGCCG

1318 base pairs

B.

GATCAGGAGAATGGAGCAAGCTCTGGGAGTTGGTGAAGGACAGAGGAGCCTGGCGTGCTGCAGT
CCATGGGGTTGCAGAGTCAGACATTACTGAGCAACCGAATGACAACAACAACAAAGATTCTTCT
AGTGAAATCCTACTCCCCAAAGTGATGGTAGTGTTAGGAGGTGGGACCTTTGAAGGGGACTAGA
TCTTAAGGCAGAGCCCTCATGATTGGAATTAGTGCCCTTATAAAAGAGACCCCAGAGAGCTCCC
TTCTGCCATGCAAGACACACAGAGAAAAGATGGCTCTCTCCTCACCAAACACCAGACCTGTTCG
TGCTGTGGTCTTGGCATTTCCATCTCTCCAGTG

353 base pairs

C.

GGCTATACTGTGGCCTGGGGAAGGGGCGTGCCCGGCGGGCGTGCCCGGCCGCCGGCGCTCACAG
GTTCTTCATGCACACCTGGCAGCGGCTGATGTGCGTGCGGATGAGGCCGGCCTTGAGCGTGTCG
GCAGAGGGCGTGCCCTGGAAGTTCTGCTCCGGGATGGTGGTCAGCCAGAAGCTGTACTTGTTGG
CGTAGTAGTCCCCGATAGAC

212 base pairs

D.
TACCTGACATGCTTCAGTGGCTTACAAGGAACAAAAAACAGGACACGTGTATACAGTAGGTACT
CATAGGTGTGCCAAGTAGGCACACATGCCTGGGCTCCATGGCACAGCTGCAAGATCAGATGTGT
CTTTGATTTCTCTCTTGAAGGCACTGGCCTCCAGGCCTGAACACACACATTGGCTTGAGAAGCG
GCACCAAGGTGGGCAGCCG
211 base pairs

E.
TACCTGACATGCTTCAGTGGAACAACAGCATCACTCATCAAACCCTGACTACGTGCCAAGTTGT
CTAGCTGTCTTCCCTCCCACCACAGTTTTGTGACAGGTACTATTATACTCCCATTTTACAGAGG
CAGGAACTGAGGCTCAGAAAGGTTCAGAGTCTTGATCAAGATCCAAGGTGGGCAGCCG
186 base pairs

Figure 4. Other sheep sequences that were obtained through attempts to clone SVCT1 and SVCT2. Primers used are underlined. 4A and 4C were the only sequences to be cloned from a PCR product that was excised from a gel. All others were cloned when PCR product was added directly to the ligation reaction. 4C was the only PCR amplification that was sequenced from SVCT2 PCR reactions. Also note that the primer for 4A, 5' CGGCTGCCCACCTTGG 3'which was supposed to be a reverse primer, as can be seen in 4D and 4E, was used as both the forward and reverse primer for this sequence.

Figures 4A, 4D, and 4E represent sequences obtained from primers that failed to amplify SVCT1. The primers 5' TACCTGACATGCTTCAGTGG 3' (forward) & 5' CGGCTGCCCACCTTGG 3' (reverse), which were based upon those used by Clark et al. (2002), were expected to yield a 1036 base pair sequence. For 4C and 4D there were thirty cycles of 1 minute at 94º C, 2 minutes at 60º C, and 3 minutes at 68º C to obtain a band of about 1300 base pairs. This band was then excised using Qiagen Midi Plasmid Prep Kit (Qiagen, Valencia, CA); 4A was the resulting sequence after subcloning. The gel extraction was then run in PCR conditions of 1 minute at 94º C, 2 minutes at 60º C, and 3 minutes at 72º C to obtain a band around 1000 base pairs. This band was then excised using Qiagen Midi Plasmid Prep Kit (Qiagen, Valencia, CA) and run in PCR conditions of 1 minute at 94º C, 2 minutes at 60º C, and 3 minutes at 72º C. The PCR product was directly subcloned; 4C and 4D were the resulting products. 4B was cloned in the subcloning reaction along with oSVCT1. 4A, 4B, 4D, and 4E were subcloned using TOPO TA Cloning® Kit For Sequencing (Invitrogen, Carlsbad, CA). 4C

was an extra PCR product that SVCT2 primers amplified. This PCR product utilized the same PCR conditions as the SVCT2 primers. It was also excised from the gel utilizing Qiagen Midi Plasmid Prep Kit (Qiagen, Valencia, CA) and subcloned using TOPO TA Cloning® Kit PCR® 2.1–TOPO® Vector (Invitrogen, Carlsbad, CA). 4C appears to be α-2 type IV collagen (COL4A2) mRNA based upon BLAST results (Altschul et al., 1997).

Discussion

$PGF_{2\alpha}$ causes luteal regression of the CL; the mechanisms by which it causes this have not been fully elucidated. The unpublished data from Gaddis and Ottobre show that $PGF_{2\alpha}$ causes an immediate decrease in progesterone synthesis in the mid-luteal phase CL, but not in the early-luteal phase (Figure 5 & 6).

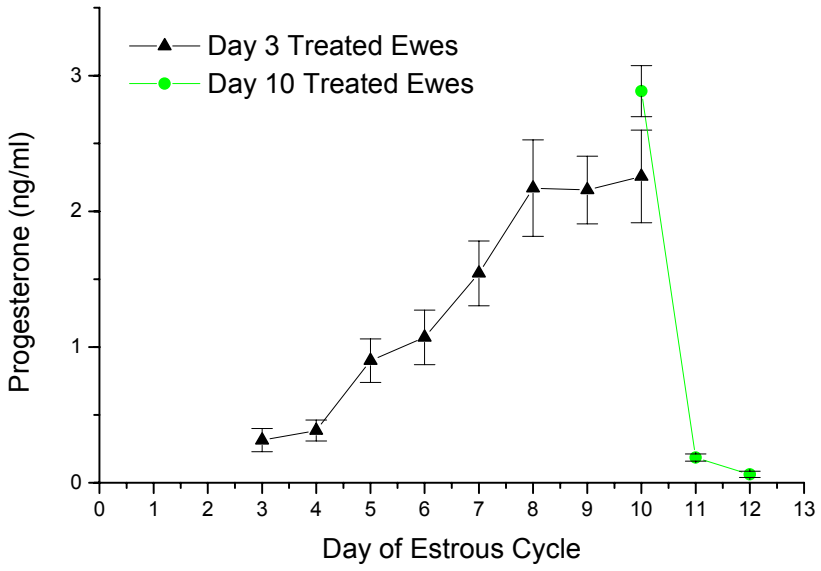


Figure 5. Concentrations of progesterone in peripheral plasma of contemporary intact sheep. $PGF_{2\alpha}$ (25mg) was administered intramuscularly on day 3 or 10 (n=7 ewes/grp). Jugular venous samples were obtained daily beginning on the day of injection and continuing for one week or the onset of estrus, whichever occurred first. Data represent means (±SE). Figure from Gaddis and Ottobre unpublished data.

Figure 5 shows this change in progesterone concentration in the peripheral plasma while figure 6 shows this change in the tissue. Progesterone concentrations in CL from the early-luteal

phase were similar at both two and twenty-four hours for $PGF_{2\alpha}$ treated tissues. In the tissues from the mid-luteal phase $PGF_{2\alpha}$-treated sheep, progesterone concentrations at two hours were lower than the control, and at twenty-four hour were lower still (Figure 6).

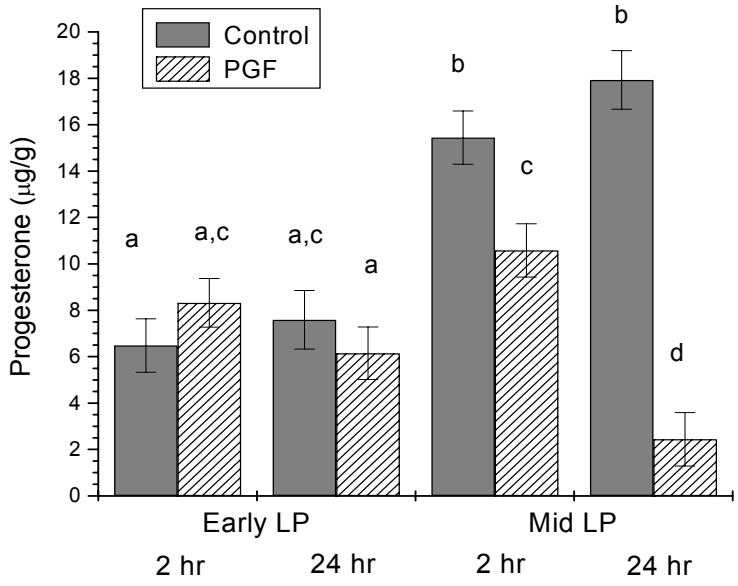


Figure 6. Progesterone concentrations (least squares mean ± SE) in corpora lutea as a function of day of cycle, $PGF_{2\alpha}$ treatment, and interval from injection to CL removal. Ewes were injected intramuscularly with $PGF_{2\alpha}$ (25mg) or saline on Day 3 or Day 10 of the estrous cycle. Corpora lutea were collected 2h and 24h after treatment. Bars with uncommon letters are significantly different ($p<0.05$, n=5-7/grp). Figure from Gaddis and Ottobre unpublished data.

The Gaddis and Ottobre unpublished data also showed the change in vitamin C concentrations in relation to the day of the cycle and to treatment with $PGF_{2\alpha}$. This is important because vitamin C is known to be important in fertility and it has been speculated that it may enhance fertility and cure some types of infertility (Kramer et al., 1933; Phillips et al., 1941; Pye et al., 1961; Luck et al., 1995; Dabrowski and Ciereszko, 2001). Figure 7 shows how the concentration of vitamin C increases between days three and ten. Figure 7 also shows how $PGF_{2\alpha}$ causes a loss of vitamin C in both the early and mid luteal phase CL, but that it is regained in the early luteal phase.

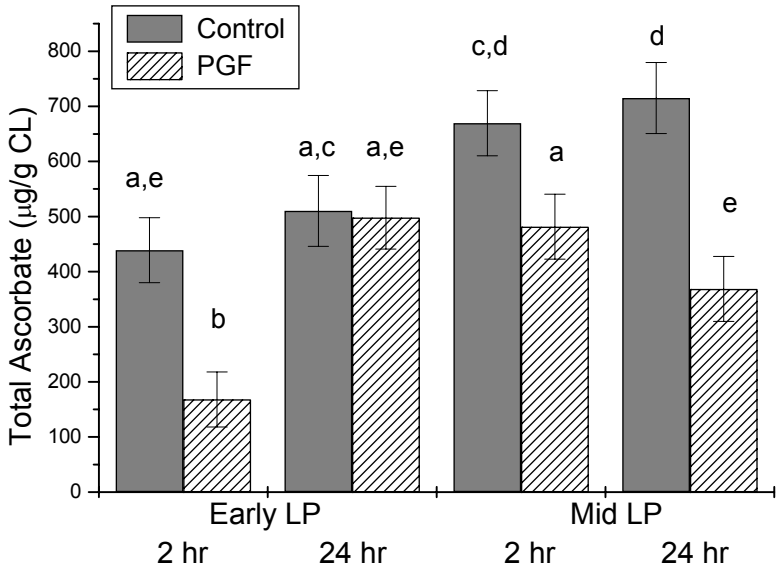


Figure 7. Ascorbate concentrations (least squares mean ± SE) in corpora lutea as a function of day of cycle, $PGF_{2\alpha}$ treatment, and interval from injection to CL removal. Ewes were injected intramuscularly with $PGF_{2\alpha}$ (25mg) or saline on Day 3 or Day 10 of the estrous cycle. Corpora lutea were collected 2h and 24h after treatment. Bars with uncommon letters are significantly different ($p<0.05$, n=5-7/grp).

This change in ability of the CL to regain vitamin C also coincides with the resistance of the CL to $PGF_{2\alpha}$. This was also confirmed in the Gaddis and Ottobre unpublished data by measuring the time required for the sheep to show estrus. The early-luteal phase CL remained functional and the sheep did not experience estrus for about fifteen more days, while the mid-luteal phase sheep experienced estrus within about two days when treated with $PGF_{2\alpha}$ (Table 4).

Treatment group	Day 3	Day 10
Average interval from $PGF_{2\alpha}$ to estrus (days)	14.71 ± 0.21	2.07 ± 0.07

Table 4. Effect of $PGF_{2\alpha}$ on interval to estrus. (mean±SE) Table from Gaddis and Ottobre unpublished data.

These data from Gaddis and Ottobre show that vitamin C can rapidly enter and leave the CL. The sodium dependent vitamin C transporters SVCT1 and SVCT2 are likely involved in actively transporting vitamin C into the luteal cells. A likely mechanism that contributes to changes in luteal concentrations of vitamin C during different stages of the estrous cycle is changes in the production of SVCT proteins. In addition, $PGF_{2\alpha}$ may interfere with the ability of SVCT proteins to transport vitamin C. Because of this, the vitamin C is lost from the tissue and antioxidants are allowed to cause damage. The early-luteal phase CL is likely expressing the genes for SVCT, which allows the transporters to be rapidly replaced, thereby resulting in re-accumulation of vitamin C. If the SVCT genes are not expressed in the mid-luteal phase, which would be possible if the proteins are stable and have a long half-life, then the vitamin C would not be reabsorbed. This scenario does not sufficiently explain the rapid loss of vitamin C from luteal tissue; however, this mechanism has not yet been described (Friedman and Zeidel, 1999; Hediger, 2002; Takanaga et al., 2004).

Another important consideration, based on data in the human (Lutsenko et al., 2004), is that there may be an SVCT2 isoform in the sheep that acts as a dominant-negative inhibitor of vitamin C transport. If this mechanism is also present in the sheep, then it could explain the inability of mid-luteal phase CL from $PGF_{2\alpha}$-treated sheep to reabsorb vitamin C. Our PCR results did not show a band corresponding to the short isoform of SVCT2. This would have been an approximately 870 base pair PCR product along with the 1196 base pair PCR product. Because PCR was performed on only a day 3 CL, the short isoform may be at a minimum concentration and therefore unobservable.

Other studies have shown that genes regulating progesterone synthesis and susceptibility to $PGF_{2\alpha}$ are regulated at the transcription level throughout the lifespan of the CL. The mRNA

for steroidogenic acute regulatory protein, which regulates progesterone synthesis, has been shown to decrease within four hours of injection of $PGF_{2\alpha}$. Concentrations of this message, reach a minimum of 36% of normal levels at twelve hours, and continue to be decreased for twenty-four hours (Juengel et al., 1995; Stocco and Clark, 1996; Niswender et al., 2000; Diaz et al., 2002). Results of studies that examined $PGF_{2\alpha}$ receptor mRNA expression varied; three studies reported consistent expression between days three, four, six, nine, ten, twelve, and fifteen (Wiepz et al., 1992; Juengel et al., 1996; Juengel et al., 1998), while two ovine studies and a bovine study using "real-time" PCR reported a decrease in mRNA expression between days ten and sixteen (Graves et al., 1995; Rueda et al., 1995a; Arosh et al., 2004); all studies agree that $PGF_{2\alpha}$ decreases $PGF_{2\alpha}$ receptor mRNA. Levels of 15-hydroxyprostaglandin dehydrogenase, which catalyzes the rate-limiting step in $PGF_{2\alpha}$ inactivation, similarly decreased from 301 ± 47 amol/μg poly(A)$^+$ RNA on day four to 17 ± 5 amol/μg poly(A)$^+$ RNA on day thirteen of the ovine estrous cycle (Silva et al., 2000). The mRNAs for protein kinase C inhibitor-1 and kinase C inhibitor protein-1, which inhibit protein kinase C, the secondary messenger system for $PGF_{2\alpha}$, have a higher concentration on day four than days ten and fifteen and are not affected by $PGF_{2\alpha}$ (Juengel et al., 1998). The mRNAs of superoxide dismutase, both the secreted and mitochondrial forms, were significantly depleted in regressing CL as compared to the functional CL (Rueda et al., 1995b). Based upon these studies, it appears that progesterone synthesizing enzymes and $PGF_{2\alpha}$ receptor mRNA are highly expressed only in the functional CL. $PGF_{2\alpha}$ can bind to the CL at any time, however $PGF_{2\alpha}$ and its second messenger system are rapidly inactivated prior to day four.

Based upon these numerous studies showing that genes involved in regulating the estrous cycle are regulated at the transcription level, we hypothesize that SVCT1 and SVCT2 will also be regulated at the transcription level. This is both due to the importance of vitamin C in the CL

and the way vitamin C concentrations change depending on the functionality of the CL. Because superoxide dismutase expression is significantly higher in the functional CL, in order to protect it from oxidative radicals, it could be likewise expected that other antioxidant regulating genes are also expressed at higher levels during times of maximal CL function (Rueda et al., 1995b). Likewise, during luteal regression, changes in the expression of vitamin C transporters could favor loss of vitamin C from the tissue and render the CL more susceptible to oxidative damage. The current data defining ovine sequences for vitamin C transporters are important in furthering studies of the regulation of SVCT proteins and vitamin C concentrations in the CL of the sheep.

Acknowledgements

We would like to acknowledge all of the advice and assistance of Charles Pretzman. This work was supported by a grant from the OARDC Research Enhancement Competitive Grants Program.

Works Cited

1. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 1997; 25:3389-3402.

2. Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J, Fortier MA. Prostaglandin biosynthesis, transport, and signaling in corpus luteum: A basis for autoregulation of luteal function. Endocrinology. 2004; 145 (5): 2551-2560.

3. Aten RF, Duarte KM, Behrman HR. Regulation of ovarian antioxidant vitamins, reduced glutathione, and lipid peroxidation by luteinizing-hormone and prostaglandin-$F_{2\alpha}$. Biology of Reproduction. 1992; 46 (3): 401-407.

4. Behrman HR, Endo T, Aten RF, Musicki B. Corpus luteum function and regression. Reproductive Medicine Review. 1993; 2:153-180.

5. Behrman HR, Preston SL. Luteolytic action of peroxide in rat ovarian cells. Endocrinology. 1989; 124: 2895-2900.

6. Bendich A, Machlin LJ, Scandurra O, Burton GW, Wayner DDM. The antioxidant role of vitamin C. Advances in Free Radical Biology and Medicine. 1986; 2 (2): 419-444.

7. Biskind GR, Glick D. Studies in Histochemistry. V. The vitamin C concentration of the corpus luteum with reference to the stage of the estrous cycle and pregnancy. The Journal of Biological Chemistry. 1936; 113:27-34.

8. Carlson JC, Wu XM, Sawada M. Oxygen radicals and the control of ovarian corpus luteum function. Free Radical Biology and Medicine. 1993; 14 (1):79-84.

9. Chen LH, Lee MS, Hsing WF, Chen SH. Effect of vitamin-C on tissue antioxidant status of vitamin-E deficient rats. International Journal for Vitamin and Nutrition Research. 50 1980; (2): 156-162.

10. Christenson LK, Farley DB, Ford SP. Evaluation of biochemical and structural changes in individual porcine corpora lutea during prostaglandin $F_{2\alpha}$-induced luteolysis with an in vitro implant system. Domestic Animal Endocrinology. 1995; 12:41-50.

11. Clark AG, Rohrbaugh AL, Otterness I, Kraus VB. The effects of ascorbic acid on cartilage metabolism in guinea pig articular cartilage explants. Matrix Biology. 2002; 21 (2): 175-184.

12. Dabrowski K, Ciereszko A. Ascorbic acid and reproduction in fish: Endocrine regulation and gamete quality. Aquaculture Research. 2001; 32 (8): 623-638.

13. Dharmarajan AM, Hisheh S, Singh B, Parkinson S, Tilly KI, Tilly JL. Antioxidants mimic the ability of chorionic gonadotropin to suppress apoptosis in the rabbit corpus luteum *in vitro*: A novel role for superoxide dismutase in regulating *bax* expression. Endocrinology. 1999; 140 (6): 2555-2561.

14. Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ, Wiltbank MC. Regulation of progesterone and prostaglandin $F_{2\alpha}$ production in the CL. Molecular and Cellular Endocrinology. 2002; 191 (1): 65-80.

15. Fomichev NI. Histochemical characteristics of corpus luteum cells. Biulleten eksperimentalnoi biologii i meditsiny. 72 (9): 107-111, 1971. (Russian)

16. Friedman PA, Zeidel ML. Victory at C. Nature Medicine. 1999; 5 (6): 620-621.

17. Gispert S, Dutra A, Lieberman A, Friedlich D, Nussbaum RL. Cloning and genomic organization of the mouse gene Slc23a1 encoding a vitamin C transporter. DNA Research. 2000; 7: 339-345.

18. Glick D, Biskind GR. The histochemistry of the adrenal gland: The quantitative distribution of vitamin C. The Journal of Biological Chemistry. 1935a; 110:1-7.

19. Glick D, Biskind GR. The histochemistry of the hypophysis cerebri: The quantitative distribution of vitamin C. The Journal of Biological Chemistry. 1935b; 110: 583-588.

20. Golumbic C, Mattill HA. Antioxidants and the autoxidation of fats. XIII. The antioxygenic action of ascorbic acid in association with tocopherols, hydroquinones and related compounds. Journal of the American Chemical Society. 1941; 63 (5):1279-1280.

21. Graves PE, Pierce KL, Bailey TJ, Rueda BR, Gil DW, Woodward DF, Yool AJ, Hoyer PB, Regan JW. Cloning of a receptor for prostaglandin $F_{2\alpha}$ from the ovine corpus luteum. Endocrinology. 1995; 136 (8):3430-3436.

22. Hediger MA. New view at C. Nature Medicine. 2002; 8 (5): 445-446.

23. Hoch-Ligeti C, Bourne GH. Changes in the concentration and histological distribution of ascorbic acid in ovaries, adrenals and livers of rats during oestrus cycles. The British Journal of Experimental Pathology. 1948; 29:400-407.

24. Horton EW, Poyser NL. Uterine luteolytic hormone: a physiological role for prostaglandin $F_{2\alpha}$. Physiological Reviews. 1976; 56:595-651.

25. Hoyer PB. Regulation of luteal regression: The ewe as a model. Journal of the Society for Gynecologic Investigation.1998; 5 (2): 49-57.

26. Juengel JL, Garverick HA, Johnson AL, Youngquist RS, Smith MF. Apoptosis during luteal regression in cattle. Endocrinology. 1993; 132 (1): 249-254.

27. Juengel JL, Meberg BM, Turzillo AM, Nett TM, Niswender GD. Hormonal-regulation of messenger-ribonucleic-acid encoding steroidogenic acute regulatory protein in ovine corpora-lutea. Endocrinology. 136 (12):5423-5429, 1995.

28. Juengel JL, Melner MH, Clapper JA, Turzillo AM, Moss GE, Nett TM, Niswender GD. Steady-state concentrations of mRNA encoding two inhibitors of protein kinase C in ovine luteal tissue. Journal of Reproduction and Fertility. 1998; 113:299–305.

29. Juengel JL, Wiltbank MC, Meberg BM and Niswender GD. Regulation of steady-state concentrations of messenger ribonucleic acid encoding prostaglandin $F_{2\alpha}$ receptor in ovine corpus luteum. Biology of Reproduction. 1996; 54: 1096–1102.

30. Kato H, Sugion N, Takiguchi S, Kashida S, Nakamura Y. Roles of reactive oxygen species in the regulation of luteal function. Reviews of Reproduction. 1997; 2: 81-83.

31. Kramer MM, Harman MT, Brill AK. Disturbances of reproduction and ovarian changes in the guinea-pig in relation to vitamin C deficiency. American journal of physiology. 1933; 106:611-622.

32. Levine M, Morita K. Ascorbic acid in endocrine systems. Vitamins and Hormones. 1985; 42:1-64.

33. Liang WJ, Johnson D, Jarvis SM. Vitamin C transport systems of mammalian cells. Molecular Membrane Biology. 2001; 18 (1): 87-95.

34. Luck MR, Jeyaseelan I, Scholes RA. Ascorbic acid and fertility. Biology of Reproduction. 1995; 52 (2): 262-266.

35. Lutsenko EA, Carcamo JM, Golde DW. A human sodium-dependent vitamin C transporter 2 isoform acts as a dominant-negative inhibitor of ascorbic acid transport. Molecular and Cellular Biology. 2004; 24 (8): 3150–3156.

36. Margolin Y, Aten RF, Behrman HR. Antigonadotropic and antisteroidogenic actions of peroxide in rat granulose cells. Endocrinology. 1990:245-250.

37. McConkey DJ, Orrenius S. Signal transduction pathways in apoptosis. Stem Cells. 1996; 14 (6): 619-631.

38. Musicki B, Aten RF, Behrman HR. Inhibition of protein-synthesis and hormone-sensitive steroidogenesis in response to hydrogen-peroxide in rat luteal cells. Endocrinology. 1994; 134 (2): 588-595.

39. Musicki B, Kodaman PH, Aten RF, Behrman HR. Endocrine regulation of ascorbic acid transport and secretion in luteal cells. Biology of Reproduction. 1996; 54 (2): 399-406.

40. Niki E. Antioxidants in relation to lipid peroxidation. Chemistry and Physics of Lipids. 1987; 44:227-253.

41. Niki E. Vitamin C as an antioxidant. World Review of Nutrition and Dietetics. 1991; 64:1-30.

42. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. Physiological Reviews. 2000; 80 (1): 1-29.

43. Niswender GD, Nett TM. 1994. Corpus luteum and its control in infraprimate species. In: Knobil E, Neill JD (eds) Physiology of Reproduction, Second Edition. Raven Press, Ltd., New York, PP781-816.

44. Packer JE, Slater TF, Willson RL. Direct observation of free radical interaction between vitamin E and vitamin C. Nature. 1979; 278:737-738.

45. Paeschke KD. [Ovulation preliminaries and ovulation. I. Generative function of the of the ovary and ascorbic acid metabolism during the ovarian cycle]. Fortschr Geburtshilfe Gynakol. 43: 1–58, 1970. (In German.)

46. Pepperell JR, Porterfield DM, Keefe DL, Behrman HR, Smith PJS. Control of ascorbic acid efflux in rat luteal cells: role of intracellular calcium and oxygen radicals. American Journal of Physiology-Cell Physiology. 2003; 285 (3): C642-C651.

47. Petroff BK, Ciereszko RE, Dabrowski K, Ottobre AC, Pope WF, Ottobre JS. Depletion of vitamin C from pig corpora lutea by prostaglandin $F_{2\alpha}$ -induced secretion of the vitamin. Journal of Reproduction and Fertility. 1998; 112(2):243-247.

48. Petroff BK, Dabrowski K, Ciereszko RE, Ottobre JS. Total ascorbate and dehydroascorbate concentrations in porcine ovarian stroma, follicles and corpora lutea throughout estrous cycle and pregnancy. Theriogenology. 1997; 47 (6): 1265-1273.

49. Phillips PH, Lardy HA, Boyer PD, Werner GM. The relationship of ascorbic acid to reproduction in the cow. Journal of dairy science. 1941; 24:153-158.

50. Pope WF, Cárdenas H. Sensitivity of sheep to exogenous prostaglandin $F_{2\alpha}$ early in the estrous cycle. Small Ruminant Research. 2004; 55: 245-248.

51. Pye OF, Taylor CM, Fontanare PE. The effect of different levels of ascorbic acid in the diet of guinea pigs on health, reproduction and survival. Journal of Nutrition. 1961; 73: 236-242.

52. Rajan DP, Huang W, Dutta B, Devoe LD, Leibach FH, Ganapathy V, Prasad PD. Human placental sodium-dependent vitamin C transporter (SVCT2): Molecular cloning and

transport function. Biochemical and Biophysical Research Communications. 1999; 262 (3): 762-768.

53. Riley JCM, Behrman HR. *In vivo* generation of hydrogen-peroxide in the rat corpus-luteum during luteolysis. Endocrinology. 1991; 128 (4): 1749-1753.

54. Rose RC, Bode AM. Biology of free radical scavengers: an evaluation of ascorbate. FASEB Journal. 1993; 7:1135-1142.

55. Rothchild I. The regulation of the mammalian corpus luteum. Recent Progress in Hormone Research. 37: 183-298, 1981.

56. Rueda BR, Botros IW, Pierce KL, Regan JW, Hoyer PB. Comparison of messenger-RNA levels for the $PGF_{2\alpha}$ receptor (FP) during luteolysis and early-pregnancy in the ovine corpus-luteum. Endocrine. 1995a; 3 (11): 781-787.

57. Rueda BR, Tilly KI, Hansen TR, Hoyer PB, Tilly JL. Expression of superoxide dismutase, catalase and glutathione peroxidase in the bovine corpus luteum: evidence supporting a role for oxidative stress in luteolysis. Endocrine. 1995b; 3 (3): 227-232.

58. Sato T, Iesaka T, Jyujo T, Taya K, Ishikawa J, Igarashi M. Prostaglandin-induced ovarian ascorbic acid depletion. Endocrinology. 1974; 95:417-420.

59. Sawada M, Carlson JC. Association of lipid peroxidation during luteal regression in the rat and natural aging in the rotifer. Experimental Gerontology. 1985; 20:179-186.

60. Sawada M, Carlson JC. Rapid plasma membrane changes in superoxide radical formation, fluidity, and phospholipase A_2 activity in the corpus luteum of the rat during induced luteolysis. Endocrinology. 1991; 128: 2992-2998.

61. Sawada M, Carlson JC. Studies on the mechanism controlling generation of superoxide radical in luteinized rat ovaries during regression. Endocrinology. 1994; 135 (4): 1645-1650.

62. Sawada M, Carlson JC. Superoxide radical production in plasma membrane samples from regressing corpora lutea. Canadian Journal of Physiology and Pharmacology. 1989; 67:465-471.

63. Sawyer DT, Chiericato G, Tsuchiya T. Oxidation of ascorbic acid and dehydroascorbic acid by superoxide ion in aprotic media. Journal of the American Chemical Society. 1982; 104:6273.

64. Silva PJ, Juengel JL, Rollyson MK, Niswender GD. Prostaglandin metabolism in the ovine corpus luteum: Catabolism of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) coincides with resistance of the corpus luteum to $PGF_{2\alpha}$. Biology of Reproduction. 2000; 63 (5): 1229-1236.

65. Stansfield DA and Flint AP. The entry of ascorbic acid into the corpus luteum in vivo and in vitro and the effect of luteinizing hormone. The Journal of Endocrinology. 1967; 39: 27-35.

66. Stocco DM, Clark BJ. Regulation of the acute production of steroids in steroidogenic cells. Endocrine Reviews 17 (3):221-244, 1996.

67. Szent-Györgyi A. Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. Biochemistry Journal. 1928; 22:1387-1409.

68. Takanaga H, Mackenzie B, Hediger MA. Sodium-dependent ascorbic acid transporter family SLC23. Pflugers Archive-European Journal of Physiology. 2004; 447 (5): 677-682.

69. Tanaka M, Miyazaki T, Tanigaki S, Kasai K, Minegishi K, Miyakoshi K, Ishimoto H, Yoshimura Y. Participation of reactive oxygen species in $PGF_{2\alpha}$-induced apoptosis in rat luteal cells. Journal of Reproduction and Fertility. 2000; 120(2):239-45.

70. Tilly JL, Tilly KI. Inhibitors of oxidative stress mimic the ability of follicle-stimulating-hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology. 1995; 136 (1): 242-252.

71. Tilly JL. The molecular basis of ovarian cell death during germ cell attrition, follicular atresia, and luteolysis. Frontiers in Bioscience. 1996; 1: D1-D11.

72. Tsai SJ, Wiltbank MC. Prostaglandin $F_{2\alpha}$ regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. Biology of Reproduction. 1998; 58 (2): 346-352.

73. Tsukaguchi H, Tokui T, Mackenzie B, Berger UV, Chen XZ, Wang YX, Brubaker RF, Hediger MA. A family of mammalian Na+-dependent L-ascorbic acid transporters. Nature. 1999; 399 (6731): 70-75.

74. Vierk JE, Hansen TR, Austin KJ, Van Kirk EA, Hess BW, Murdoch WJ. Inhibition by tocopherol of prostaglandin-induced apoptosis in ovine corpora lutea. Prostaglandins & Other Lipid Mediators. 1998; 56 (5-6): 265-276.

75. Wang HP, Dutta B, Huang W, Devoe LD, Leibach FH, Ganapathy V, Prasad PD. Human Na+-dependent vitamin C transporter 1 (hSVCT1): primary structure, functional characteristics and evidence for a non-functional splice variant. Biochemica et Biophysica Acta-Biomembranes. 1999; 1461 (1): 1-9.

76. Wang YX, Mackenzie B, Tsukaguchi H, Weremowicz S, Morton CC, Hediger MA. Human vitamin C (L-ascorbic acid) transporter SVCT1. Biochemical and Biophysical Research Communications. 2000; 267 (2): 488-494.

77. Wiepz GJ, Wiltbank MC, Nett TM, Niswender GD, Sawyer HR. Receptors for prostaglandins $F_{2\alpha}$ and E_2 in the ovine corpora lutea during maternal recognition of pregnancy. Biology of Reproduction. 1992; 47:984-991.

78. Winkler BS, Orselli SM, Rex TS. The redox couple between glutathione and D ascorbic-acid – A chemical and physiological perspective. Free Radical Biology & Medicine. 1994; 17: 333–349.